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Complexity of nutrient use efficiency in plants

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Chapter 2

Localization of sulfate uptake and pH changes at roots of intact *Brassica pekinensis* seedlings under sulfur deprivation by using H⁺-selective microelectrodes

Martin Reich, Marten Staal, Luit J. De Kok, J. Theo M. Elzenga

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Abstract

Proton selective micro-electrodes were used to determine sulfate uptake by roots of intact plant seedlings. The response of H^+ fluxes to sulfate addition proved to be a good measure for sulfate uptake by the sulfate/ H^+ co-transport system. H^+ influx and increase in root surface pH was much higher in sulfur-deprived seedlings than in seedlings grown with sufficient sulfate. The opposite was true for the response of H^+ fluxes to nitrate addition. By using this method sulfate uptake could be mapped along the root axis which revealed higher uptake rates in mature regions. Sulfur-deprived roots showed a lower root surface pH which correlated strongly with the response to sulfate addition. A possible contribution of this component to a higher sulfate uptake capacity under sulfate deprivation was further tested by using the fungal toxin fusaric acid which permanently activates the plasma membrane H^+ -pumping ATPase. Application of fusaric acid lowered the pH of sulfur sufficient roots to the level of sulfur-deprived roots, indicating a higher activity of the H^+ -ATPase under sulfur deprivation rather than a higher abundance.

2.1 Introduction

Sulfate is taken up by plant roots via a H^+ -coupled symport over the plasma membrane (Hawkesford et al., 1993; Smith et al., 1995; Hawkesford et al., 2003) and serves as the main source of sulfur to plants. The regulation of sulfate transport is well studied on the molecular, biochemical and whole plant level (see *e.g.* Saito, 2000; Buchner et al., 2004; Hopkins et al., 2005; Koralewska et al., 2008; Rouached et al., 2008) but knowledge on the local distribution of uptake along the root axis is still scarce. The few studies on localization of sulfate fluxes along roots are rather indirect via localization of the sulfate transporter genes using in situ hybridization or GFP fusion (Takahashi et al., 1997, 2000; Yoshimoto et al., 2002). Electrophysiological studies applying ion selective micro-electrodes offer the possibility to measure ion uptake in real-time and with high temporal and spatial resolution (Plas-sard et al., 1999; Newman, 2001). Localization along the root axis of different plant species have been conducted for fluxes of for example nitrate, ammonia, potassium and H^+ (Henriksen et al., 1990; Taylor and Bloom, 1998; Garnett et al., 2001; Rubinigg et al., 2002; Chen et al., 2005; Staal et al., 2011) and gave important insight into the acquisition of these nutrients in relation to nutrient supply and developmental processes.

Application of ion-selective microelectrodes relies on highly sensitive and selective ion exchangers. Up to now, no ion exchanger with satisfying properties is available for sulfate. Instead H^+ selective micro-electrodes were used in the present study to determine and localize sulfate uptake activity along roots of intact seedlings of Chinese cabbage, a plant that is known for its high sulfur need and sulfate content (Koralewska et al., 2008). Because sulfate transporters are H^+ /sulfate co-transporters (Hawkesford et al., 1993), sulfate addition should cause an immediate response of H^+ fluxes proportional to the coupled influx of sulfate. To test this assumption we compared fluxes at roots of plants that had been grown with sufficient sulfate in the medium to fluxes of sulfur-deprived plants. As in the latter situation the sulfate transporters are known to be strongly upregulated (*e.g.* Buchner et al., 2004), larger H^+ fluxes in response to sulfate addition were expected. As an additional verification of the method $\text{Mg}(\text{NO}_3)_2$ was added to the roots instead of MgSO_4 . Nitrate uptake is known to be depressed under sulfate deprivation and the response of H^+ fluxes in response to $\text{Mg}(\text{NO}_3)_2$ is expected to be higher at sulfate sufficient than in sulfate-deprived roots. After these verifications the method was used to localize sulfate uptake along the root axis of sulfur-sufficient and deprived roots. At the same time, differences in root surface pH were assessed.

2.2 Material and Methods

Plant material

Seeds of Chinese cabbage (*Brassica pekinensis* Rupr., cv. Michico) were germinated on filter paper, moistened with tap water and placed in dark at 21°C. After four days, seedlings were placed in a climate controlled room with a day/night temperature of 21°C/18°C ($\pm 1^\circ\text{C}$), a photoperiod of 14 h and a photon flux of ca. $340 \mu\text{mol m}^{-2} \text{s}^{-1}$. Seedlings were positioned in a way to allow the roots growing into the solution. The seedlings were kept under these conditions for another 3 days (Fig. 2.1) before being transferred from the filter paper to in the 13 liter containers with aerated 25% Hoagland nutrient solution (pH 5.9), consisting of 1.25 mM $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 1.25 mM KNO_3 , 0.25 mM KH_2PO_4 , 11.6 μM H_3BO_3 , 2.4 μM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.24 μM $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.08 μM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.13 μM $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ and 22.5 μM Fe^{3+} -EDTA containing either 0.5 mM (+S) or 0 mM (-S) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. The pH was controlled and adjusted every two to three days with diluted HCl.

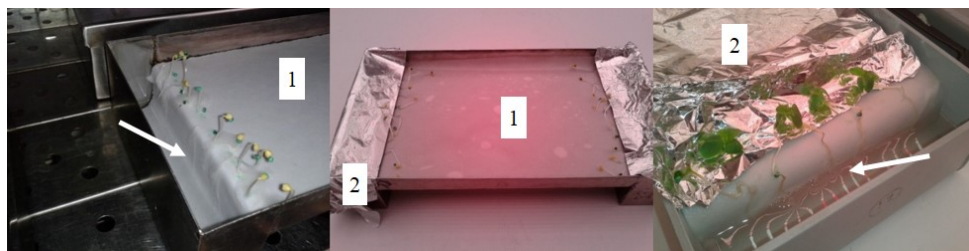


Figure 2.1: Seeds were germinated on filter paper (1) in germination trays close to the edge of the basin. In this way most of the initiating roots grew into the solution (arrow). When placed in the climate room the filter paper was covered with aluminum foil (2) to avoid growth of algae and fungi.

Plant preparation and ion flux experiments using the MIFE technique

As root excision changes nutrient absorption (Bloom and Caldwell, 1988; Shabala et al., 2009) intact seedlings were used in this study. Prior performing an ion flux experiment, a plant was taken from the climate room and placed in a 25 ml petri dish and the primary root was mounted carefully in such a way that it was easily accessible with the ion selective electrode. The shoot was stabilized at the edge of the petri dish. Thereafter the root was covered with a low salt measuring solution (MS; containing 200 μM MgCl_2 , 100 μM KCl and 100 μM CaCl_2). During the whole measurement the shoot of the plant was enclosed in a small cabinet and supplied with LED light with a photon flux of ca. $250 \mu\text{mol m}^{-2} \text{s}^{-1}$, while the roots were not illuminated. The MS

was continuously exchanged by a perfusion system with a perfusion rate of ca. 3 ml min^{-1} (Fig. 2.2). The tip of the electrode was then moved to the root



Figure 2.2: Seedlings were gently fixed in petri dishes (1) with their roots submerged in the measuring solution. The shoot was transferred into a custom-made, light-proof enclosure (2) without damaging leaves or stem. With an LED spotlight (3) the shoot was illuminated during the whole measurement. The reference electrode (4) was submerged in the reference solution and connected with the measuring solution via a salt bridge. The inlet of the perfusion system was positioned as far from the measuring position as possible (black arrow). Everything was arranged in a way that the microelectrode (white arrow) could access the root.

surface by using a three dimensional micromanipulator and an inverted microscope. Steady fluxes were recorded for at least ten minutes before the MS was exchanged with the treatment solution. The total incubation time before a measurement was at least one and not more than two hours, depending on the time it took to record a stable flux. Net fluxes of H^+ were measured using H^+ -selective electrodes with the MIFE technique (Microelectrode Ion Flux Estimation; Shabala et al., 1997; Vreeburg et al., 2005; Lanfermeijer et al., 2008). Microelectrodes were pulled from borosilicate glass capillaries (GC150-10; Harvard Apparatus) and silanized with tributylchlorosilane (Fluka 90974). The H^+ -selective electrodes were back filled with 15 mM of NaCl and 40 mM of KH_2PO_4 and front filled with Hydrogen Ionophore II (Cocktail A; Fluka 95297). Only electrodes with a response of $> 53 \text{ mV per pH unit}$ (pH range 5.1–7.8, $r^2 > 0.998$) were used for measurements. The reference electrode, filled with 300 mM KCl was placed in a separate compartment electrically connected with the measuring chamber via a salt bridge consisting of 300 mM $(\text{NH}_4)_2\text{SO}_4$ in 2% (w/v) agar. Prior to flux and pH recording the electrode was brought carefully to the defined distance of $10 \mu\text{m}$ from the root surface. To ensure a maximum response of H^+ -fluxes to sulfate and a minimum response to its accompanying cation it was decided to apply sulfate in the form of MgSO_4 as the uptake Mg^{2+} is known to be slower than one of the other cations, especially in the presence of Ca^{2+} and K^+ (Moore et al., 1961; Schimansky, 1981).

Time series recordings

The microelectrode was positioned at the beginning of the differentiated zone, where the first root hairs started to appear (usually 3-5 mm from the root tip). At this position the response in time of root surface pH and H^+ fluxes to

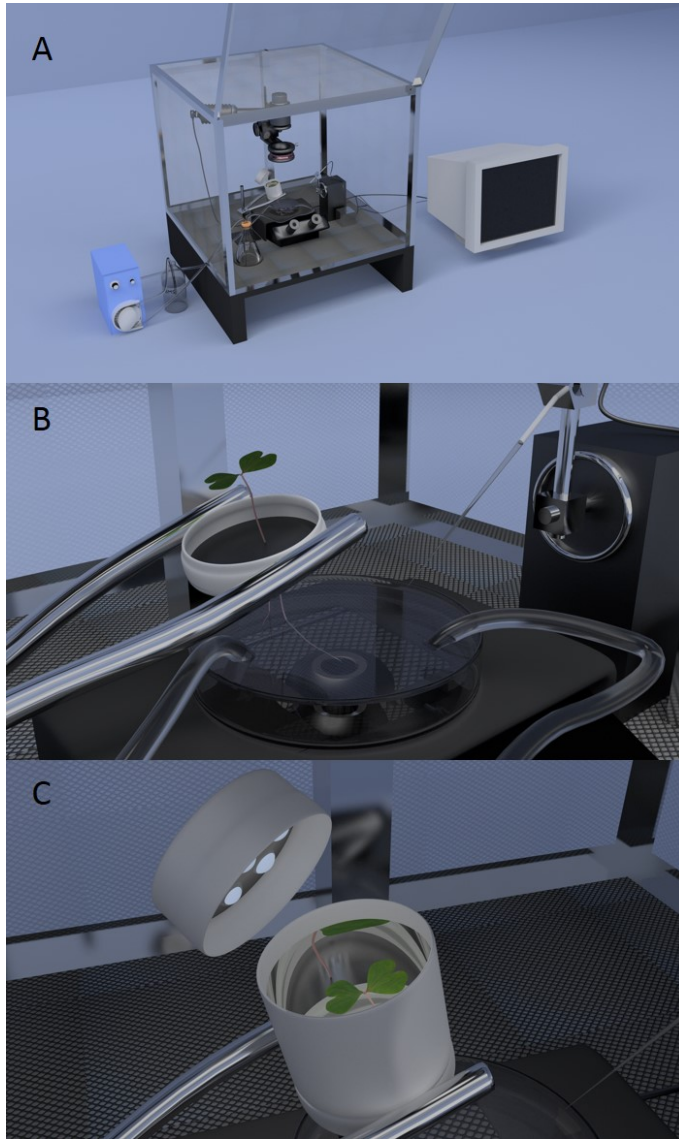


Figure 2.3: A: The measurement took place in a Faraday's cage in which an inverted microscope was installed to position the electrode at the root surface. The amplifiers, the pump for the perfusion system (left) and the PC for data read-out (right) were located outside of the cage. B: The plant shoot was enclosed in the lower part of a small chamber and the root was immobilized in the petri dish which was filled with the measuring solution. The electrode was mounted on a 3D-micromanipulator. Inlet and outlet of the perfusion system were placed at the edges of the dish. C: The plant shoot was fully enclosed and illuminated during the measurement (Visualization by Markus Reich).

the addition of different compounds via the perfusion system were examined. Care was taken not placing the tip of the electrode above an initiating root hair. Instead it was positioned between the very first root hairs. During the incubation phase before a time series measurement the position of the electrode and its distance to the root surface was surveyed and corrected if necessary. For the time series recording sulfur sufficient (Control) seedlings and seedling which were sulfur deprived for one to five days (-S) were taken.

Localization of H^+ -fluxes along the root axis of sulfur-sufficient and sulfur-deprived seedlings in response to sulfate addition

To localize the H^+ -flux and pH changes along the root axis, a root profile was recorded from the root tip until 7 mm towards the base in 0.5 mm increments between 0-5 mm and 1 mm increments between 5 and 7 mm. A control profile was recorded after one to two hours incubation in the sulfate-free measuring solution. Subsequently the solution was exchanged to one of the same kind but containing 100 μM $MgSO_4$ and after 30 minutes a second profile had been recorded. At each position fluxes and pH were recorded for 3-4 minutes and averaged. The profiling included the meristematic, the elongation and the beginning of the differentiated zone. For the root profiles sulfur-sufficient (Control) seedlings and seedling which have been sulfur deprived for three days were taken (-S).

Fusicoccin experiment

For the fusicoccin (FC) experiment excised roots were used instead of seedlings, first because FC is too costly to be applied in the large volume of the perfusion system and second because the response of the H^+ -ATPase to FC is expected to be immediate. Roots were placed in 1 ml measuring chambers. After an incubation time of ca. one hour 10 μM fusicoccin (Sigma, F0537) was added and the solution was firmly mixed.

2.3 Results

Response of H^+ -fluxes and surface pH at roots of sulfur-sufficient and sulfur-deprived plants to the addition of sulfate and nitrate

A clear response of surface pH, *i.e.* the concentration of H^+ at the root surface, could be observed if 100 μM $MgSO_4$ was added to the roots via the perfusion system (Fig. 2.1). The response was much more pronounced at sulfate-deprived roots with pH increasing ca. 0.2 units which corresponds to a decrease in $[H^+]$ of ca. 0.5 μM . Alkalization was also observed if 50 μM

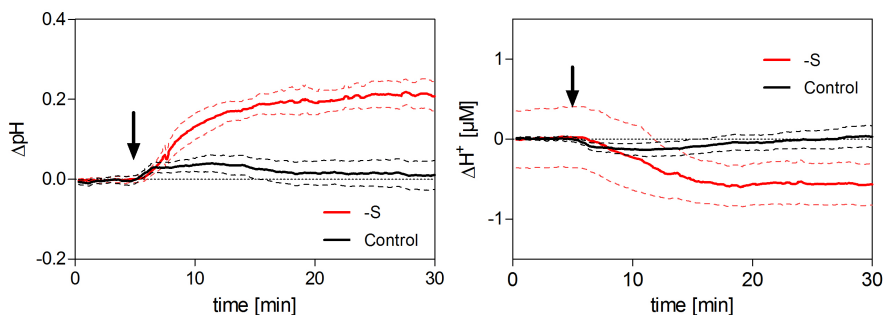


Figure 2.4: The change in response of proton fluxes and surface pH at roots of intact seedlings to sulfate addition (indicated by the arrow) over time. Plants were either grown with sufficient sulfate (Control) or without sulfate (-S). Positive values refer to an influx. Mean values are shown, the broken lines indicate the SE. (Control $n = 12$; -S $n = 15$)

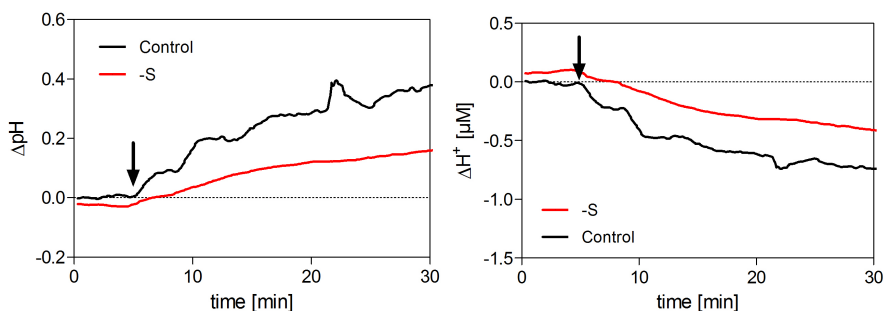


Figure 2.5: Tentative results of the change in response of proton fluxes and surface pH to nitrate addition (indicated by the arrow). Plants were either grown with sufficient sulfate (Control) or without sulfate (-S). Positive values refer to an influx (exemplary measurement).

$\text{Mg}(\text{NO}_3)_2$ was added to the measuring solution but the sulfur status of the plants had an opposite effect, with sulfur sufficient plants showing a greater response (ca. 0.3 pH units) than sulfur-deprived plants (ca. 0.1 pH units; Fig. 2.4).

Localization of sulfate uptake along the root axis

H^+ fluxes showed a high variation while surface pH was more stable, as it is the result of net H^+ fluxes over a longer time period. For this reason the results for pH were more consistent and robust. A response to MgSO_4 after 30 min of addition was present at roots of sulfur-deprived seedlings between 3.5 and 7 mm away from the root tip but absent in the first 3 mm which covered the meristematic and the beginning of the elongation zone (Fig. 2.5).

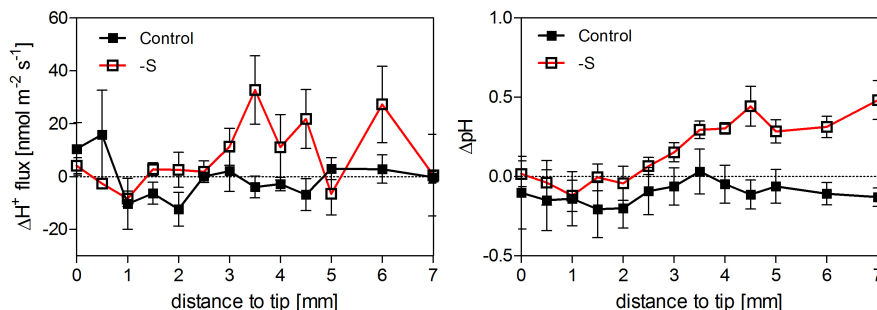


Figure 2.6: Root profiles of proton fluxes and surface pH in response to sulfate addition at intact roots of sulfur sufficient (Control) and three days sulfur-deprived (-S) seedlings. Indicated are the changes of fluxes and pH in response to sulfate addition at the same position are shown. Positive values refer to an influx. Data represent the mean (\pm SE; $n = 3$).

No changes of surface pH were observed in sulfur sufficient plants.

Surface pH at roots of sulfur-deprived and sufficient seedlings

A lower surface pH at roots of sulfur deprived plants compared to sufficient plants was observed during the measurements prior to any compound addition. There was a linear relationship between the concentration of H^+ before and the change in concentration of H^+ in response to sulfate addition ($r^2 = 0.86$; $p < 0.0001$; Fig. 2.7). Additional experiments revealed that the difference in surface pH was also apparent if measurements were undertaken in the growth medium and therefore was not an artefact of the incubation in the measuring solution (Fig. 2.7).

Fusicoccin experiment

Adding fusicoccin (FC) to the roots resulted in an immediate H^+ efflux and a consequent decrease in pH. Thirty minutes after addition, the pH at roots of sulfur-sufficient seedlings was approximately at the same level as the one of sulfur deprived ones (Fig. 2.8). The response of H^+ -flux and surface pH to FC in a typical experiment is presented in Fig. 2.9.

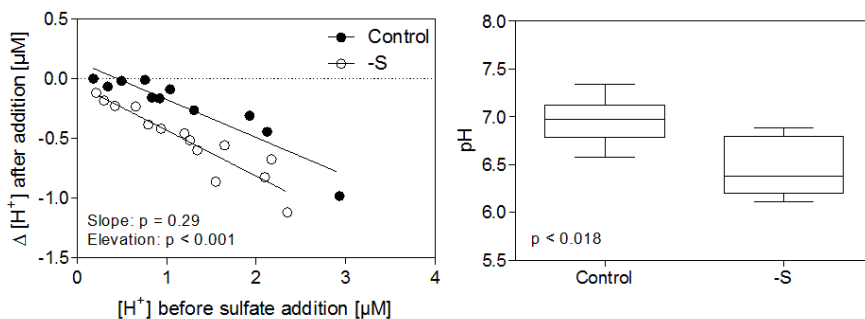


Figure 2.7: Roots of sulfur deprived seedlings (open symbols) showed a lower surface pH than roots of sulfur sufficient seedlings (closed symbols) and the concentration of $[H^+]$ at the root surface correlated with the peak response of $[H^+]$ to addition of $100 \mu M$ $MgSO_4$. The elevation of the two linear regression significantly differs (p-value shown). The differences in root surface pH were also measured in the growth medium (25 % Hoagland). Data presented as boxes with a 5-95 percentile and whiskers (unpaired Student t-test; Control: $n = 6$; -S: $n = 5$).

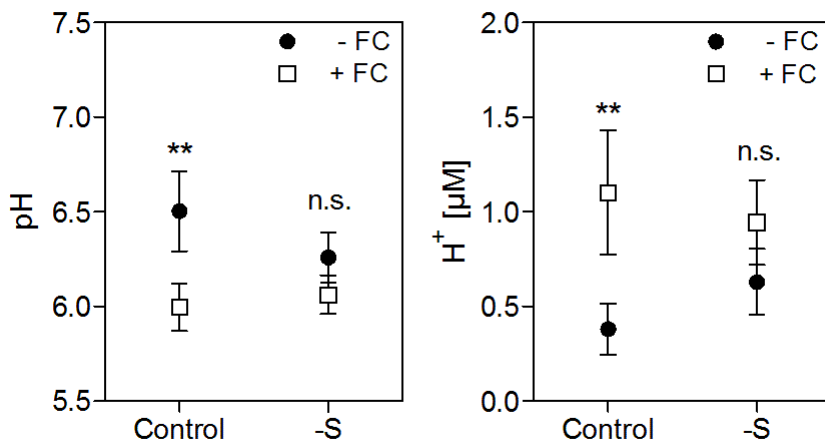


Figure 2.8: Average of root surface pH and $[H^+]$ of sulfur-sufficient (Control) and sulfur-deprived (-S) seedlings 10 minutes before (closed symbols) and peak values after (open symbols) the addition of $10 \mu M$ FC. Data represent the mean (\pm SE; ** = $p < 0.01$; One-way-ANOVA; Control: $n = 3$; -S: $n = 4$)

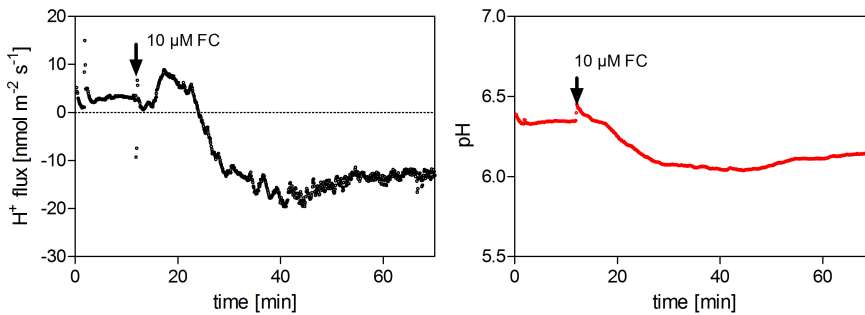


Figure 2.9: Over-time response of H^+ -flux and root surface pH following FC addition (indicated by the arrow). Negative values correspond to an efflux.

2.4 Discussion

H^+ -selective microelectrodes as a tool to determine sulfate uptake

The results in this study show that H^+ -selective microelectrodes are a convenient tool to determine and localize the uptake of sulfate at roots. Two main lines of experimental evidence support this. First, the de-repression of the sulfate uptake system under sulfur deprivation is clearly reflected by the pH changes at the root surface in response to sulfate addition (Fig. 2.4). The relatively weak alkalization at sulfur-sufficient roots disappeared after ca. 10 minutes while the strong alkalization at sulfur-deprived roots reached a stable plateau. Second, addition of nitrate in pre-liminary experiments resulted in a converse response of sufficient and deprived roots, if compared to sulfate addition. This was expected, as sulfur-deprived plants usually down-regulate nitrate uptake (Clarkson et al., 1989; Prosser et al., 2001). The use of H^+ -selective microelectrodes appears therefore a reliable method to probe plant roots for differences in sulfate uptake.

In a case study, we applied this method to map sulfate uptake along the first millimeters of the roots of seedlings. We found that no active sulfate uptake was apparent in the meristematic zone and beginning of the elongation zone (0-3 mm), 30 minutes after sulfate addition (Fig. 2.6). Up to date, the only localization studies concerning sulfate transport at roots of intact plants aimed on localizing transcripts of the genes encoding for the sulfate transporters (*e.g.* Takahashi et al., 1997, 2000; Yoshimoto et al., 2002). *In situ* hybridization analyses showed gene expression in the root cap, the epidermal layer, in root hairs and along the root cylinder. Abundance of mRNA transcripts is, however, not necessarily reflecting the final localization and

activity of transport proteins. Many post-translational modifications and cellular circumstances determine if and how many ions a transport-protein finally gates through the membrane and most of these mechanisms are still poorly understood or not even discovered, yet. The tissue- and cell-specific topology of sulfate uptake remains an open field of research. The results of our analysis of the actual fluxes at intact roots suggest that active sulfate uptake takes place in the more differentiated root zones rather than in the meristematic and elongation zone.

Lower root surface pH under sulfur deprivation correlates with sulfate uptake

Fig. 2.7 shows that sulfur-deprived plants respond stronger to sulfate addition than sulfur-sufficient plants, indicated by the significant difference in elevation ($p < 0.001$). This is most likely representing the higher abundance of sulfate transporters. Additionally, a strong correlation was found between the H^+ concentration at the root surface prior and after the addition of sulfate (Fig. 2.7). This dependency of sulfate uptake on the H^+ gradient at the root plasma membrane confirms the suggested SO_4^{2-}/H^+ -symport function of sulfate transporters (Hawkesford et al., 1993). Additionally it raises the question whether a lower external pH and a consequently steeper H^+ -gradient could have a physiological function to increase the uptake capacity under sulfur deprivation (Fig. 2.10). Indeed, a lower root surface pH was also found at seedlings grown in Hoadland solution instead of the measuring medium (Fig. 2.7).

While the role of sulfate transporters in sulfate uptake has been studied intensively, the involvement and potential co-regulation of the plasma membrane ATPase is not yet investigated. Active rhizosphere acidification has well been described and characterized in plants deprived of phosphate and iron (De Vos et al., 1986; Neumann and Römheld, 1999). The uptake of these nutrients is mostly limited by their mobility in the soil solution, which is increased at low pH. Sulfate in contrast, if present, is highly mobile over a wide pH range. A lower root surface pH under sulfate deprivation could also be of passive nature, caused by an altered cation-anion uptake balance (Haynes, 1990): no sulfate and probably less nitrate is taken up and, consequently, also less protons. Our experiments rather suggest, however, an involvement of the activity of the plasma membrane H^+ -ATPase: incubation of roots with fusicoccin, a permanent activator of the plasma membrane H^+ -ATPase (Johansson et al., 1993), led to a decrease of the surface pH at sulfur sufficient roots to the same level of sulfur-deprived roots (Fig. 2.8). Therefore, it appears that the root plasma membrane H^+ -ATPases are not more abundant under sulfur deprivation but in a more activated state. Sulfate uptake has

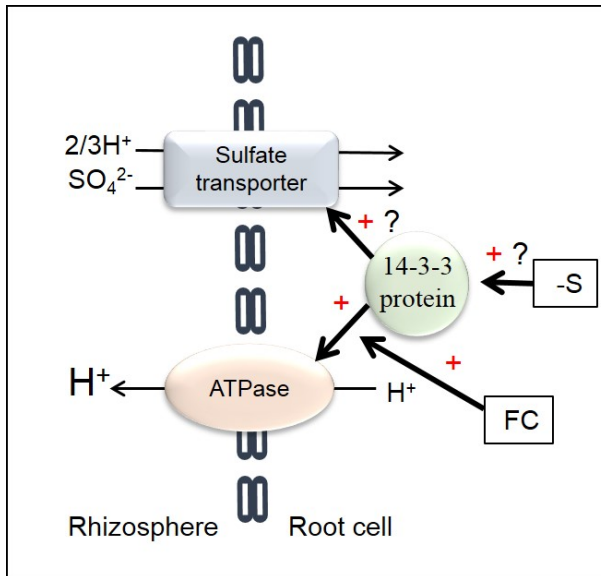


Figure 2.10: Lower rhizosphere pH as an adaptive response to sulfur deprivation (-S)? Isoforms of the 14-3-3 protein were shown to stimulate the H^+ -ATPase and to interact with the sulfate transporter 1.2 in a yet unknown way (Shin et al., 2011) and might act as a coordinator to increase sulfate uptake under sulfur deprivation. Fusicoccin (FC) permanently stabilizes the binding of 14-3-3 to the H^+ -ATPase and can therefore be used to achieve a maximum activation.

been shown to be in a repressed state if sulfate or other sulfur sources are sufficiently abundant, which enables a quick de-repression if sulfate gets limiting (Clarkson and Saker, 1989; Herschbach and Rennenberg, 1994). The exact cellular and molecular signals involved need still to be elucidated and some authors actually found discrepancies between the sulfate uptake capacity and the expression level of the genes encoding for sulfate transporters (Koralewska et al., 2009). Deeper understanding of the regulation on the protein and membrane level is likely to explain such observed discrepancies. The plasma membrane H^+ -ATPase as driver of H^+ -coupled sulfate uptake (Fig. 2.10) might be involved in this regulation and increase sulfate uptake in addition to a higher transcript level of the sulfate transporter genes. Because the plasma membrane H^+ -ATPase is building up the proton gradient that is utilized also by all other transport systems, a coordination with the uptake of other nutrients would be needed. A candidate player for such a general regulatory function is the 14-3-3 protein. Isoforms of this protein were shown to interact not only with the plasma membrane H^+ -ATPase (Jahn et al., 1997; Bunney et al., 2002) but also with numerous proteins of nitrogen, phosphorous and sulfur metabolism, including the sulfate transporter Sultr1.2 which is responsible for primary sulfate uptake at the root plasma membrane (Shin et al., 2011). Being “spiders in a web of phosphorylation” (De Boer et al., 2013), 14-3-3 proteins could be central coordinators of the complex network of different proteins responsible for ion homeostasis and the uptake of nutrients. Further clarification is needed whether a lower rhizosphere pH under sulfur deprivation is an active response or rather a passive consequence of re-

duced anion uptake. However, active or passive, a lower pH has the potential to increase sulfate uptake Fig. 2.10). Future studies should aim to further integrate cellular and electrophysiological techniques to elucidate the coordination of H^+ -ATPase activity and nutrient status. Additionally, methods to assess the nutrient status or gene expression level of homogenized plant tissue reached their limit in explaining the complex processes under nutrient deficiency. Roots are composed of highly diversified tissues, quickly changing their morphology to adapt to changes in nutrient availability with newly formed roots having a different physiology than older ones. Cellular heterogeneity of ion transport is still widely understudied but crucial to understand how root development and morphology determine and control nutrient uptake. Connecting the morphological changes under sulfur deficiency with the cellular responses of the sulfate uptake system should be a future aim to understand the whole plant regulation of sulfate uptake.

More advanced techniques are needed to account for this morphological and physiological complexity on small scales, such as high-resolution imaging techniques or ion-selective microelectrodes which are perfectly suited to localize ion uptake on small scales and should be used more often, also in combination with molecular techniques. As long as there are no highly selective sulfate electrodes available, H^+ -electrodes are a reliable alternative to study sulfate uptake. Localization of sulfate uptake at different kinds of tissues or the characterization of specific mutants are possible applications. However, as the determination relies on the response of $[H^+]$ to the addition of a sulfate pulse, continuous measurements of sulfate fluxes, for example in response to mutual signal compounds, remain difficult.

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